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(57) Abstract

The present invention discloses enzymes having xylanase considerable activity at a pH of 9.0 and a temperature of 70 °C. The enzymes are obtainable from deposited strains which are related to alkaliphilic Bacilli. The enzymes are suited for use in paper and pulp production processes.

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Alkalitolerant xylanases

Technical field

The present invention relates to novel microorganisms and to novel enzymes. More specifically the enzymes are alkalitolerant xylanases. These xylanases are obtainable from gram-positive, alkalitolerant microorganisms. The xylanases are applicable under conditions used in the paper and pulp industry i.e. pH = 9 and T = 70°C.

Background of the invention

Xylan is a component of plant hemicellulose. Xylan consists of 1,4-glycosidically linked β-D-xylose. Usually xylans have side chains containing xylose and other pentoses, hexoses and uronic acids.

In the paper production process the bleaching of pulp is an important step. Schematically the steps used in the pulp treatment in paper and pulp industry is performed as follows:
Pulp is treated at pH 10-12 at 80°C to remove most of the lignin in the so-called oxygen delignifying step. The remaining pulp contains 2-5% of lignin. This lignin gives the pulp the brown color. Subsequently, the pulp is bleached in a multistage bleaching process. In this bleaching chemicals such as chlorine, chlorine dioxide, hydrogenperoxide and/or ozone are used to obtain a pulp for high quality paper.

Chlorine and chlorine-containing chemicals are often used to remove lignin, which is responsible for the brownish color of the pulp. Use of the indicated chemicals leads to the formation of dioxin and other chlorinated organic compounds. These compounds form a threat to the environment and

there is a growing tendency to omit the use of chemicals giving rise to similar waste products.

This has prompted a tendency to develop chlorine-free processes; total chlorine free (TCF) and elementary chlorine-free (ECF). In these processes hydrogen peroxide or ozone is used for bleaching.

It has been found that the introduction of an enzymatic step in the paper and pulp preparation process has several advantages.

Xylanases have been found to be very useful in the paper and pulp processing. Xylanases have been reported to increase the extractability of lignins from the pulp. Xylanases are mostly used after the oxygen delignifying step.

Xylanases cleave the hemicellulose chain linking the lignin to the cellulose chain. After xylanase treatment the lignin is more easily removed in the subsequent steps.

Therefore the use of xylanases leads to a reduction of the consumption of active chlorine in prebleaching of 25-30%. This reduction of chlorine does not afflict the quality parameters of the resulting paper (Viikari et al. 1986. Proc. of the third Int. Conf. Biotechnology in Pulp and Paper Ind., Stockholm, p.67-69 and Bajpai and Bajpai. 1992. Process Biochemistry. 27 : 319-325).

The xylanase treatment also reduces the need for other chemicals in the bleaching process.

The use of xylanases from fungal sources in bleaching of kraft pulp has been reported. The pH and temperature optima of these enzymes are : pH = 3-5 and T = 30-50°C. These values are not ideal for the use in the bleaching process where the prevailing conditions are pH $\geq$ 9 and temperature $\geq$ 70°C.

Xylanases from bacterial origin, with higher pH and/or temperature optima have also been reported for use in the bleaching process. Some of these are the following:

Bacillus pumilus (pH = 7-9, T = 40°C, Nissen et al., 1992. Progress in Biotechnology 7 : 325-337), Dictyoglomus thermophilum (pH = 6-8, T = 70°C, European patent application EP 0 511 933), B.stearothermophilus T-6 (pH = 9.0, T = 65°C, Shoham, Y. et al. (1992) Biodegradation 3, 207-18),

B.stearothermophilus (pH = 9, T = 50°C, WO 91/18976) and Thermoanaerobacter ethanolicus (68°C, Deblois and Wiegel.1992. Progress in Biotechnology 7 : 487-490).

Even though most of the above cited xylanases show activity at pH ≥ 9 and temperature ≥ 70°C, their effectiveness under industrial application conditions (i.e. during the bleaching of pulp), in terms of e.g. increased brightness of the pulp is only limited and can vary significantly (see e.g. WO 91/18976, highest increase in pulp brightness at pH 9 and 50°C is only 0.5 % ISO brightness).

Summary of the invention

The present invention relates to xylanases having considerable activity at pH 9.0 and at a temperature of 70°C, and which is characterized in that the xylanase is obtainable from a microorganism of which the 16S ribosomal DNA sequence shares more than 92 % identity with the 16S ribosomal DNA sequence of strain DSM 8721 as listed in SEQ ID NO 20.

The present invention also relates to xylanases having considerable activity at pH 9.0 and at a temperature of 70°C, and characterized in that the xylanase is obtainable from a microorganism selected from the group consisting of the strains deposited under the following deposition numbers: CBS 666.93, 667.93, 669.93, and 673.93.

The present invention further relates to xylanases having considerable activity at pH 9.0 and a temperature of 70°C further characterized in that the xylanase produces an increase in % ISO brightness of soft-wood pulp over non-enzymatically treated pulp of at least 1.0, preferably an increase in % ISO brightness of soft-wood pulp between 1.5 and 5.0, in an ECF pulp bleaching process wherein the enzyme treatment of the pulp is carried out at a pH of 9.0 at a temperature of 65°C.

The present invention also relates to xylanases having considerable activity at pH 9.0 and a temperature of 70°C further characterized in that the xylanase produces an increase in % ISO brightness of soft-wood pulp over non-enzymatically treated pulp of at least 1.0, preferably an increase in % ISO brightness of hard-wood pulp between 1.2 and 3.0, in an ECF pulp bleaching process wherein the enzyme treatment of the pulp is carried out at a pH of 9.0 at a temperature of 65°C.

Detailed description of the invention

The present invention relates to microorganisms which have been isolated from soil and water samples collected in the environment of alkaline soda lakes in Kenya, East-Africa. These microorganisms have been characterized as being alkaliphilic, Gram-positive and belonging to the genus Bacillus (see below).

The microorganisms have subsequently been screened using a xylan-agar diffusion assay. Strains which showed a clearing zone in this test were isolated as potential xylanase producing strains.

The strains were grown at pH 10, and T = 45°C. After centrifugation the culture broth was tested for xylanase activity in an assay at pH = 9 and T = 80°C (Example 2).

Eight different strains were found to produce xylanase activity under the indicated conditions. These microorganisms have been deposited at the Centraal Bureau voor de Schimmelcultures in Baarn, the Netherlands under

deposition number CBS 666.93, 667.93, 668.93, 669.93, 670.93, 671.93, 672.93, 673.93.

Most of these strains have been send to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) for an independent identification using comparisons of 16S ribosomal DNA sequences as described by Nielsen et al. (1994, FEMS Microbiol. Lett. 117, 61-65). On the basis of this sequence comparison the eight strains can be assigned to the genus Bacillus and are most related to B.alcalophilus (DSM 485^T). The sequence comparison further shows that the eight strains fall into two groups. The first group is very similar or almost identical to DSM 8721 and comprises strains 1-16-2, 1-25-2, and 1-43-3 (CBS 670.93, 671.93, 672,93, respectively). The second group is most related to DSM 8718 and comprises strains 2-47-1, 2-M-1, 1-47-3 and 2-26-2 (CBS 666.93, 667.93, 669.93 and 673.93), respectively. The classification of the deposited strains into these two groups is confirmed by xylanase zymograms.

Surprisingly, we have found that the xylanases obtainable from the first group of strains, i.e. the strains most related to DSM 8721 (comprising 1-16-2, 1-25-2, and 1-43-3) show a superb performance in the bleaching of pulp. This performance is exemplified by the increased brightness of both soft-wood and hard-wood pulp when treated with the enzymes of the present invention and is most pronounced on softwood pulp. In this respect, the performance of the xylanases obtainable from most of the strains in the second group, i.e. the group related to DSM 8718, is much less, although the xylanases obtainable from strain 1.47.3. shows the best performance on hard-wood pulp as compared to the other strains. The increase in brightness obtained with the enzymes of the present invention is at least 1.0, expressed as Δ Final ISO Brightness over the non-enzymatically treated control pulp. Preferably the brightness increase in the case of soft-wood pulp is between 1.5 and 5.0, and in the case of hard-wood pulp between 1.2 and 3.0.

The present invention discloses enzymes having xylanase activity and having a considerable xylanase activity at pH 9 and at a temperature of about

70°C. Said enzymes are obtainable from the deposited strains. Said enzymes are also obtainable from mutants and variants of the deposited strains.

With the expression 'considerable activity' is meant that the enzymes of the present invention have at pH=9, 40% of the activity they possess at pH=7, preferably this is 60%, more preferably about 80%. In a most preferred embodiment of the present invention the activity of the xylanase is higher at pH=9 than at pH=7.

The present invention also discloses a process for the production of subject xylanases, which can be developed using genetic engineering. As a first step the genes encoding the xylanases of the present invention can be cloned using λ-phage (expression-) vectors and E.coli host cells. Alternatively, PCR cloning using consensus primers designed on conserved domains may be used. On the basis of homology comparisons of numerous xylanases a distinction in different classes has been proposed (Gilkes et al., 1991, Microbiol. Rev. 55, 303-315). For each class specific conserved domains have been identified. Class F and class G xylanases can be identified based on this determination. DNA-fragments in between two conserved domains can be cloned using PCR. Full length clones can be obtained by inverse PCR or by hybridization cloning of gene libraries. Expression of some of the genes encoding the xylanases of the present invention in E.coli is shown to give an active protein. Said proteins are active at pH 9 at a temperature of 70°C.

After a first cloning step in E.coli, a xylanase gene can be transferred to a more preferred industrial expression host such as Bacillus or Streptomyces species, a filamentous fungus such as Aspergillus, or a yeast. High level expression and secretion obtainable in these host organisms allows accumulation of the xylanases of the invention in the fermentation medium from which they can subsequently be recovered.

The present invention further relates to a process for the preparation of xylanases obtainable from the deposited strains and having considerable activity at a pH of 9 at a temperature of 70°C. The process comprises cultivation of the deposited microorganisms or recombinant host

microorganisms expressing genes encoding the xylanases of the present invention in a suitable medium, followed by recovery of the xylanases.

The enzymes of the present invention have been shown to have a considerable activity on oat spelt xylan and on birchwood xylan.

The enzymes of the present invention have further been tested for their bleaching activities. The enzyme preparations, xylanases, are capable of delignifying wood pulp at a temperature of at least 80°C and a pH of at least 9. The expression "wood pulp" is to be interpreted broadly and is intended to comprise all kinds of lignocellulosic materials. The enzymes of the present invention can be used immediately after the oxygen delignifying step in the paper and pulp preparation process described above. Preferably, the enzymes are used before the oxygen delignifying step. In this step the lignin concentration is much higher therefore the effect of the application of the xylanase is much larger.

The enzymes of the present invention have been tested for their activity on both hardwood and softwood pulps. Apart from the kappa reduction, also the increase in brightness has been determined on two types of pulp, both soft-wood and hard-wood kraft pulp in ECF bleaching experiments. It follows that the increased brightness produced by the xylanases of the present invention would also allow to reduce the amount of bleaching chemicals while achieving the same brightness as obtained without the use of enzymes.

Furthermore, the inventions relates to the applications of the enzyme preparations of the invention, particularly to a process in which wood pulp is treated with said enzyme preparations according to the invention, and a wood pulp and a fluff pulp treated with the enzyme preparations according to the invention.

The invention further relates to paper, board and fluff pulp made from a wood pulp treated with the enzyme preparations according to the invention.

The enzyme preparations of the present invention have further been shown to have a low cellulase activity.

EXAMPLE 1

Isolation of alkali- and thermotolerant xylanases

Samples

Soil and water samples were collected in the environments of alkaline soda lakes in Kenya, East Africa.

Screening for xylanase producing microorganisms

Two methods were applied for the isolation of xylanase-producing microorganisms:

i) The soil and water samples were suspended in 0.85% saline solution and directly used in the xylan-agar diffusion assay.

ii) The soil and water samples were incubated in a xylan containing liquid minimal medium or GAM-medium for 1 to 3 days at 45, 55 and 70°C respectively. Cultures that showed bacterial growth were analyzed for xylanase activity using the xylan-agar diffusion assay.

Media

The minimal medium (pH 9.7) used in the xylan-agar diffusion assay and the enrichment procedure, consisted of KNO_3 1%, Yeast extract (Difco) 0.1%, KH_2PO4 0.1%, $MgSO_4.7H_2O$ 0.02%, Na_2CO_3 1%, NaCl 4% and a mixture (0.05% each) of four commercially available xylans [Xylan from oat spelts (Sigma X-0376), Xylan from birchwood (Sigma X-0502), Xylan from oat spelts (Serva 38500), Xylan from larchwood (ICN Biochemicals 103298)]. For solidification 1.5% agar is added.

The complex medium (GAM) used for enzyme production consisted of Peptone (Difco) 0.5%, Yeast extract (Difco) 0.5%, Glucose.H_2O 1%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.02%, Na_2CO_3 1%, NaCl 4%. The pH is adjusted to 9.5 with 4M HCl after which 1% Xylan (Serva) is added.

Xylan-agar diffusion assay

Cell suspensions in 0.85% saline solution were plated on Xylan containing minimal medium. After incubation for 1 to 3 days at 45 and 55° C respectively, the strains that showed a clearing zone around the colony were isolated as potential xylanase producing microorganisms.

Isolation of alkali- and thermotolerant xylanase producing strains

Strains that showed clearing zones in the agar diffusion assay were fermented in 25 ml GAM-medium in 100 ml shake flasks in an Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA), at 250 r.p.m. at 45°C for 72 hours. Xylanase activity was determined in the culture broth at pH 9 and 80°C (Example 2).

Isolation of crude enzyme preparations

Shake flask fermentations were carried out in 2 l erlenmeyer flasks containing 500 ml GAM-medium. The flasks were incubated in an orbital incubator at 250 r.p.m. at 45°C for 48 to 96 hours. The cells were separated from the culture liquid by centrifugation (8000 rpm). The cell-free culture liquid was concentrated by ultrafiltration, using an Amicon Stirred Cell Model 8400 with YM5 filter.

EXAMPLE 2

Characterization of alkali- and thermotolerant xylanases

Analytical methods

Assays for xylanase activity are performed using modified procedures of the Sumner assay (J. Biol.Chem. 1921. 47 5-9).

Procedure 1

Xylanase activity on Oat Spelts xylan

A test tube is filled with 200 µl 4% Oat spelts xylan suspension, 600 µl aliquots of cell-free culture broth (Example 1) diluted in the appropiate buffer. The test tube is incubated in a waterbath for 15 minutes. After the incubation, 7.2 ml DNS (Dinitrosalicylic acid) reagent is added. The mixture is heated in a waterbath at 100°C for 10 minutes. After heating the mixture the test tube is cooled on ice. The absorbance is measured at 575 nm. To eliminate the background absorbance of the enzyme samples a control experiment was executed as follows: a tube with substrate incubated under the same conditions as the test tube. After incubation 7.2 ml DNS and the enzyme preparation is added (in this order). One unit of xylanase (xU) activity is defined as the amount of enzyme producing 1 µmol of xylose from xylan equivalent determined as reducing sugar per minute.

Actual measuring conditions were pH 7, 9 and 70 and 80 °C. The buffers were Phosphate pH 7 and Borate/KCl pH 9. The results are shown in table 1 as relative activity.

Table 1: Relative xylanase activities on Oat Spelts xylan

RELATIVE XYLANASE ACTIVITY ON OAT SPELTS XYLAN			
	strain	70 °C	
Nr	number	pH 7	pH 9
1	1-47-3	100	82
2	2-47-1	100	51
3	2-m-1	100	67
4	1-16-2	100	55
5	1-25-2	100	40
6	2-16-1	100	63
7	1-43-3	100	48
8	2-26-2	100	59

The strains indicated in Tables 1, 2 and 3 as 1 to 8 have been deposited under the following deposition numbers;

2-47-1 = CBS 666.93, 2-m-1 = CBS 667.93

2-16-1 = CBS 668.93, 1-47-3 = CBS 669.93

1-16-2 = CBS 670.93, 1-25-2 = CBS 671.93

1-43-3 = CBS 672.93, 2-26-2 = CBS 673.93

Procedure 2

Xylanase activity on Birchwood xylan

The same method as described in procedure 1 is used. Instead of a 4% Oat Spelts xylan suspension a 4% Birchwood xylan suspension is used. The test conditions were: pH 7 and 9 and 70 and 80 ° C, respectively. The results are shown in table 2.

Table 2: Relative xylanase activities on Birchwood xylan

Nr	strain	pH 7 70°C	pH 9 70°C	pH 7 80°C	pH 9 80°C
1	1-47-3	100	72	100	10
2	2-47-1	100	80	100	9
3	2-M-1	100	90	100	8
4	1-16-2	100	40	100	42
5	1-25-2	100	24	100	65
6	2-16-1	100	74	100	11
7	1-43-3	100	23	100	55
8	2-26-2	100	69	100	18

EXAMPLE 3

Delignification assay at 70°C and 80°C

Kappa assay

The kappa assay's were performed according to the TAPPI T236 protocol with some modifications. The enzyme solution was added at a dose of 10 xU/g pulp (based on Oat spelts xylan for the pulp nb 1 and based Birchwood

xylan for pulps 2 and 3) (dry weight) and incubated for 2 hours at pH 9, 70 and 80 °C. The control, was pulp incubated for the same period under the same conditions without enzyme addition. Tree different pulps were used:

1] Kraft softwood pulp

2] Kraft softwood pulp after oxygen delignification

3] Kraft hardwood pulp after oxygen delignification

Pulp properties (nb 2 and 3):

	Hardwood Birch 80%	Softwood spruce, 20% pine
Brightness, % ISO	50.8	35.8
Kappa number	11.0	16.7
Viscosity, dm^3/kg	979	1003
Calcium, ppm	1900	2600
Copper, ppm	0.3	0.6
Iron, ppm	5.1	11
Magnesium, ppm	210	270
Manganese, ppm	25	70

The difference between the kappanumber with enzyme addition and the kappanumber without enzyme addition is called the kappa reduction and is a value for delignification. The kappa reductions are shown in table 3A.

Table 3A: Kappa reductions at pH 9 and 70 °C and 80 °C

Nr	Strain Number	pH 9 70°C Softwood kraft pulp (nb 1) kappa red	pH 9 70°C Softwood O2 delig (nb 2) kappa red	pH 9 80°C Hardwood O2 delig (nb 3) kappa red
1	1-47-3	1.7	0.3	
2	2-47-1	2		
3	2-M-1	2		
4	1-16-2	1.8		
5	1-25-2	1.6	1.1	0.5
6	2-16-1	0.4		
7	1-43-3	1.1	1.2	1
8	2-26-2	0.5		

blanks were not determined.

Delignification assay at 60°C

Kappa assay

The kappa assay's were performed according to the Tappi T236 protocol with some modifications. The enzyme solution was added at a dose of 10 xU/g pulp (based on birchwood xylan) (dry weight) and incubated for 2 hours at pH 9 , 60°C. The control, was pulp incubated for the same period under the same conditions without enzyme addition. Two different pulps were used:

- Kraft hardwood pulp after oxygen delignification (nb 2).
- Kraft softwood pulp after oxygen delignification (nb 4).

Pulp properties (nb 2 and 4)

	Hardwood Birch 80 %	Softwood
Brightness, % ISO	50.8	40.0
Kappa number	11.0	10.1
Viscosity, dm^3/kg	979	940
Calcium, ppm	1900	1800
Copper, ppm	0.3	0.3
Iron, ppm	5.1	5.2
Magnesium, ppm	210	250
Manganese, ppm	25	35

The difference between the kappanumber with enzyme addition and the kappanumber without enzyme addition is called the kappa reduction and value for delignification. The kappa reductions are shown in table 3B.

Table 3B Kappa reductions at pH 9 and 60°C.

Nr	Strain number	Softwood O2 delig kappa red	Hardwood O2 delig kappa red.
1	1-47-3	0.0	
2	2-47-1	0.9	
3	2-M-1	0.5	0.6
4	1-16-2	1.1	0.7
5	1-25-2	0.9	0.2
6	2-16-1	0.7	0.2
7	1-43-3	1.1	
8	2-26-2	0.7	

EXAMPLE 4

Cellulase activity

Assay's for cellulase activity were performed using a modified procedure of the PAHBAH (parahydroxybenzoicacid hydrazide) assay (Anal. Biochem. 1972. 47 : 273-279)

0.9 ml 0.5% CMC (carboxymethylcellulose) is incubated with 0.1 ml diluted enzyme preparation and incubated for 60 minutes at pH 9 and 70 °C. after the incubation 3 ml PAHBAH reagent (10 ml 5% PAHBAH in 0.5M HCl was mixed with 40 ml 0.5M NaOH = PAHBAH reagent) is added and the reaction mixture is heated for 5 minutes at 100 °C. After cooling on ice the absorbance is measured at 420 nm. To eliminate the background absorbance of the enzyme samples a control experiment was executed as follows: the CMC was incubated for 30 minutes at pH 9, 70 °C and the enzyme solution is added after adding of the PAHBAH reagent. One cellulase unit (cU) is defined as the quantity of enzyme necessary to produce one µMol glucose per minute (using CMC as substrate) and is related to the xylanase activity. All strains tested showed a cellulase activity less than 10 mU CMCase per unit of xylanase.

EXAMPLE 5

Cloning of xylanase genes and fragments thereof

Chromosomal DNA was isolated from strains mentioned in Example 2 according to methods described (Maniatis et al, Cold Spring Harbor Laboratory Press, 1989). Genomic libraries were prepared for each of these selected strains using the ZAP Express® cloning system available from Stratagene. The host/vector system was used according to the instructions of the supplier (Catalog # 239212, june 30, 1993). For construction either partial Sau3A digest ligated into the BamH1 site or randomly sheared DNA supplied with EcoR1 linkers ligated into the EcoR1 site were used.

Recombinant phages were transformed into plasmid vectors as recommended by the supplier. These plasmid vectors were tested for expression of xylanase using RBB xylan indicator plates.

Positive colonies were isolated and tested for production of xylanase using the following medium:

Production medium:

4 x L B C :

20 g yeast extract

40 g Bacto trypton

10 g NaCl

4 g casaminoacids

fill up to 1 liter with demineralized water ass 0.25 ml antifoam and sterilize 20' at 120 °C. Colonies are grown during 24 hr at 30 °C under vigourous shaking.

The enzyme was isolated using a heat shock method (10' at 65°C) to lyse the cells. Xylanase activity was measured as described above.The results of the tests of individual clones are summarized in Table 4.

Table 4. Xylanase activities of cloned xylanases expressed in E.coli.

Strain	Clone	Production level (U/ml)
1-47-3	KEX101	0.6
	KEX106	23.7
	KEX107	17
2-M-1	KEX202	<0.2
	KEX203	4.0
1-43-3	KEX301	40
	KEX303	1.1
	KEX304	1.8
2-26-2	KEX401	12
	KEX402	12
	KEX403	43
	KEX404	33
	KEX405	<0.2
	KEX406	17
	KEX407	110
	KEX408	0.8
	KEX409	36

It can be concluded that all clones produce xylanase. Although the variability in production level might be due to cloning of partial gene fragments, it most

probably can be regarded as a reflection of the diversity of xylanase genes present within the inserts.

EXAMPLE 6

Characterization of selected xylanase encoding inserts

The DNA insert of xylanase producing clones can be characterized by DNA sequencing. The insert of KEX106 was analysed and a gene encoding the alkalitolerant xylanase was identified. The DNA sequence of the gene is shown in SEQ ID NO 1.

A comparison of the amino acid sequence of the encoding protein (SEQ ID NO 2) revealed an homology to xylanase protein sequences, i.e. 93 % [Hamamoto et al., 1987, Agric. Biol Chem., 51, 953-955].

The amino acid sequence of xylanases of the present invention can therefore share an identity with the amino acid sequence of SEQ ID NO 2 of higher than 93 %, preferably the identity is at least 95 %, more preferably the identity is at least 98 %, and most preferably more than 99 %.

EXAMPLE 7

Identification and cloning of internal fragments of genes encoding alkalitolerant xylanases

As an alternative method to the screening of gene libraries we have worked out a method based on PCR cloning. On the basis of a comparison of numerous xylanase sequences we have designed consensus oligonucleotide primers encompassing conserved sequence boxes. Two types of primers have been designed. One set of primers is for the F-type of xylanase and one set is for the G-type of xylanases.

The following consensus primers have been constructed:

FA: 5′ CAC ACT/G CTT/G GTT/G TGG CA 3′: forward primer, consensus box 1 (SEQ ID NO 3)

FB: 5′ CAT ACT/G TTT/G GTT TGG CA 3′: forward primer, consensus box 1 (SEQ ID NO 4)

FR: 5′ TC/AG TTT/G ACC/A ACG/A TCC CA 3′: reverse primer, consensus box 2 (SEQ ID NO 5)

Primers FA and FB bind to the same consensus box, but due to slight differences in the nucleotide sequence they exhibit complementary specificity.

PCR conditions were as follows: [94 °C, 1 min], [50 °C, 1 min] and [72 °C, 1 min] for 30 cycles. Fragments originating from amplification with F-type primers were purified on agarose gel and subcloned. Subsequently the DNA sequence was determined.

G_{AF}: 5′ GAA/G TAT/C TAT/C ATT/C/A GTN GA : forward primer, consensus box 1 (SEQ ID NO 6)

G_{BF}: 5′ GAA/G TAT/C TAT/C GTN GTN GA : forward primer, consensus box 1 (SEQ ID NO 8)

G_{AR}: 5′ CG/TN ACN GAC CAA/G TA : reverse primer consensus box 2 (SEQ ID NO 7)

G_{BR}: 5′ CG/TN ACA/G CTC CAA/G TA : reverse primer consensus box 2 (SEQ ID NO 9)

G_{CR}: 5′ CCR CTR CTK TGR TAN CCY TC : reverse primer consensus box 3 (SEQ ID NO 10)

PCR conditions were as follows: [94 °C, 1 min], [40 °C, 1 min] and [72 °C, 1 min] for 30 cycles.

The first PCR with G-primers was performed with primers constructed on box 1 and box 3. The resulting mixture of fragments of different sizes were subsequently purified from agarose gel (250-340 bp) and subjected to a

second round of PCR, now using primers from box 1 and box 2. Unique fragments were amplified and subcloned. The blunt-end repair of the PCR fragments was performed in the PCR mix by adding 0.5 mM ATP (Boehringer Mannheim), 10 u T4 DNA kinase (BRL), 1 u T4 DNA polymerase (BRL) and incubation at 37 °C for 1 hour. The mixture was purified using the PCR extraction kit from Qiagen. The fragment was ligated into the pUC18xSmaI (CIAP) vector obtained from Appligene according to Maniatis. E. coli HB101laqIq was transformed with the ligation mixture using electroporation. The DNA sequence of a number of individual clones was determined.

From the analysis it has become apparent that the selected strains harbor several different xylanase genes, some of which may be cloned by the F-type consensus primers and other which may be cloned by the G-type of primers. As an example several different internal xylanase fragments originating from strains 1-43-3, 1-47-3, 1-M-1, 2-26-2 (all F-type) and 1-43-3 and 1-25-2 (all G-type) are depicted in the sequence listings (see Table 5).

Table 5.

Strain	Consensus primers used	Sequence listing
1-43-3	F-type	SEQ ID NO 11
1-47-3	F-type	SEQ ID NO 12
2-26-2	F-type	SEQ ID NO 13
2-M-1	F-type	SEQ ID NO 14
1-25-2	G1-type	SEQ ID NO 15
1-43-3	G1-type	SEQ ID NO 16
1-43-3	G2-type	SEQ ID NO 17

The cloned internal fragment are subsequently used as a specific probe to isolated the cloned gene fragments from the lambdaZAP gene library using

stringent hybridisation conditions. All cloned genes can be isolated using this method.

The method is especially advantageous for those genes that do not express well from their native gene regulatory signals in E.coli, since these genes would escape from detection in the method described in example 5. Using subcloning methods and DNA sequence analysis the complete genes encoding the various alkalitolerant xylanases can be isolated and equipped with expression signals for production in E.coli.

EXAMPLE 8

Further characterization of xylanase clones

With the aid of both the consensus primers and specific primers a further characterization of the clones mentioned in example 5 was performed. It became apparent that there is a clustering of xylanase genes on several of the cloned inserts. On the basis of this inventory single genes were subcloned in expression vectors for both E. coli and Bacillus subtilis. Expression of monocomponent xylanases was obtained upon transformation into E.coli and Bacillus respectively. The Bacillus expression system was based on the PlugBug® technology [ref1]

EXAMPLE 9

Characterization of selected G-type xylanase encoding insert

The insert of clone KEX301 was analysed and an open reading frame encoding a G-type xylanase was identified. The sequence of this ORF is given in SEQ ID NO 18 and the derived amino acid sequence for the xylanase in SEQ ID NO 19. A search for homologous genes within the EMBL database (release 39, version 2) showed that the sequence of G1 xylanase is unique. No DNA homology of more than 68 % was detected. Also the protein sequence was compared to the database sequences. The closest homology (72 %) was found with a xynY xylanase sequence (Yu et al. 1993, J. Microbiol. Biotechnol. 3, 139-145).

The amino acid sequence of xylanases of the present invention can therefore share an identity with the amino acid sequence of SEQ ID NO 19 of at least 72 %, preferably the identity is at least 80 %, more preferably the identity is at least 90 %, still more preferably the identity is at least 95 %, and most preferably more than 99 %.

ref1 : Quax, W.J. et al, 1993, in Industrial Microorganisms: Basic and Applied Molecular Genetics, ASM, Washington D.C., p143.

EXAMPLE 10

Pulp bleaching experiments with supernatants from deposited strains

All experiments were elemental chlorine free (ECF) bleaching with a XwDED bleach sequence. Enzyme treatments on pulp were for two ours at pH 9.0 and 65°C. To ensure proper temperature throughout the experiment the pulp has been heated in the microwave to 65°C before adding enzyme. Experiments were run at a pulp consistency of 10 %, which was adjusted by adding pH adjusted tap water. A summary of the ECF bleaching data for xylanase containing culture supernatants of the deposited strains is shown in Table 6.

Table 6. Brightness increase expresses as Δ Final ISO Brightness over the non-enzymatically treated control for the supernatants of the deposited strains and for the reference Cartazyme GT 630 (Sandoz).

Strain/Enzyme	Softwood	Hardwood
1.43.3	3.55	1.45
1.47.3	1.45	1.99
2.47.1	1.8	1.55

1.25.2	3.15	1.45
2.M.1	0	0.4
1.16.2	1.55	0.5
2.26.2	0	0.9
GT 630	0	0

Before each bleaching experiment every enzyme containing supernatant was assayed for xylanase activity at pH 9.0, 65°C. In the bleaching experiments 2 xylanase units per gram of oven dried pulp were used for each supernatant. Supernatant activities were determined the same day the enzyme bleaching stage was run.

EXAMPLE 11

Pulp bleaching with cloned xylanase genes expressed in E.coli

Xylanases obtained from three of the E.coli clones expressing cloned xylanase genes obtained from the deposited strains were tested in pulp bleaching experiments as described in Example 10. The E.coli clones were cultured as described in Example 5. Recombinant enzyme was isolated from the E.coli bacteria in one of three ways:

1. Whole lysate

   In this case, the whole cell culture (cells + spent growth medium) was harvested. Cells were disrupted by sonication followed by heat at 65°C for 10 minutes. The lysates were then clarified by centrifugation.

2. Cell pellet

   Cells were separated from spent medium by centrifugation. Cell pellets were resuspended at 10 ml/g wet weight in 50 mM Tris/HCl, pH 7.0 buffer. The cell suspension was then sonicated and heated as described for "whole lysate".

3 Culture supernatant

Spent growth medium was separated from whole cells by centrifugation. The clarified medium was then diafiltered (tangential flow, 10,000 MWCO membrane) to reduce the total volume and exchange the liquid 50 mM Tris/HCl, pH 7.0 buffer.

The results of the bleaching experiments are shown in Table 7.

Table 7. Pulp bleaching with cloned xylanase genes expressed in E.coli

Parental strain	Clone #	source of enzyme	Δ Final ISO Brightness*	
			Soft-wood	Hard-wood
1-47-3	KEX 106	whole lysate	decrease	decrease
1-43-3	KEX 301	whole lysate	3.2	n.d.**
		cell pellet	3.4	0.7
		cell pellet	n.d.	1.0
		culture sup.	3.6	1.5
1-43-3	KEX 303	cell pellet	3.2	1.6
		culture sup.	n.d.	1.0

* over non-enzymatically treated control

** n.d. = not determined

EXAMPLE 12

Identification of the deposited strains

Most of these strains have been send to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) for an independent identification using comparisons of 16S ribosomal DNA sequences as described by Nielsen et al. (1994, FEMS Microbiol. Lett. 117, 61-65). The results of this identification are provided in Table 8. On the basis of this sequence comparison the eight strains can be assigned to the genus Bacillus and among the known Bacilli, they are most related to B.alcalophilus (DSM 485^T).

The sequence comparison further shows that the eight strains fall into two groups. The first group is very similar or almost identical to DSM 8721 and comprises strains 1-16-2, 1-25-2, and 1-43-3 (CBS 670.93, 671.93, 672,93, respectively). The second group is most related to DSM 8718 and comprises strains 2-47-1, 2-M-1, 1-47-3 and 2-26-2 (CBS 666.93, 667.93, 669.93 and 673.93), respectively.

The xylanases of the invention are preferably obtainable from the first group of strains, i.e. the strains most related to DSM 8721 (comprising 1-16-2, 1-25-2, and 1-43-3). The xylanases of the present invention are therefore obtainable from Bacillus strains of which the 16S ribosomal DNA sequence shares at least 92 % identity with strain DSM 8721, preferably the identity is at least 93.3 %, more preferably at least 96.6 %, still more preferably at least 99 %, and in the most preferred embodiment the identity is 100%.

Tabel 8. 16S rDNA sequence similarities of the deposited strains to some alkaliphilic Bacilli

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. 1-9A-1(93-509)	-																						
2. 1-43-3(93-510)	99.2	-																					
3. 2-47-1(93-511)	89.4	88.6	-																				
4. 2-26-2(93-512)	89.5	88.9	99.6	-																			
5. 1-25-2(93-513)	100.0	98.6	89.3	89.3	-																		
6. 1-47-3(93-514)	89.6	89.7	99.9	99.5	89.7	-																	
7. 1-16-2(93-515)	100.0	99.2	89.5	89.5	100.0	89.8	-																
8. 2-M-1(93-516)	89.6	89.5	99.9	99.8	89.5	99.7	89.7	-															
9. *B. alcalophilus*	91.3	90.7	95.8	96.1	91.6	95.6	91.4	95.8	-														
10. *B. colmii*	88.0	87.4	92.4	92.1	87.2	92.0	88.0	92.0	93.4	-													
11. DSM 8714	89.6	88.6	92.6	92.2	89.3	92.5	89.7	92.4	94.9	91.9	-												
12. DSM 8715	90.4	89.4	94.4	94.3	90.6	94.3	90.5	94.3	96.4	94.0	94.8	-											
13. DSM 8716	89.6	88.7	93.3	92.8	89.6	93.0	89.8	92.9	95.0	92.0	96:0	94.8	-										
14. DSM 8717	90.3	89.4	93.7	93.4	89.6	93.3	90.4	93.5	95.6	93.1	96.8	94.2	96.1	-									
15. DSM 8718	88.8	88.0	98.9	99.3	89.5	99.0	88.9	99.1	96.3	93.7	93.6	96.0	94.0	93.8	-								
16. DSM 8719	87.9	87.0	92.8	92.6	87.2	92.5	97.8	92.6	93.0	97.2	90.7	92.8	91.7	92.2	93.5	-							
17. DSM 8720	95.6	94.6	92.5	92.4	95.6	92.5	95.6	92.5	93.3	91.7	90.9	93.7	91.5	91.6	93.0	92.1	-						
18. DSM 8721	100.0	99.4	90.6	90.2	100.0	90.5	100.0	90.2	93.2	91.1	91.4	93.3	91.9	91.7	92.3	91.0	96.6	-					
19. DSM 8722	90.0	89.1	92.3	92.1	90.3	92.0	90.1	92.1	94.7	92.1	94.6	94.8	94.4	94.4	94.0	91.3	92.1	92.1	-				
20. DSM 8723	87.5	86.7	93.3	92.8	86.8	92.9	87.5	93.0	93.3	97.6	91.3	93.1	92.6	92.9	93.8	98.4	92.0	90.8	91.4	-			
21. DSM 8724	91.3	90.3	95.7	96.1	91.7	95.6	91.4	95.8	99.9	93.3	94.8	96.2	95.0	95.5	96.2	92.9	93.3	93.2	94.6	93.2	-		
22. DSM 8725	89.6	89.2	94.6	94.3	90.0	94.3	89.7	94.6	98.1	93.1	94.4	95.9	94.8	94.8	95.4	92.4	93.0	92.2	94.5	92.8	90.1	-	
23. *B. subtilis*	89.2	88.3	91.9	91.7	89.9	91.9	89.2	91.7	92.6	93.9	91.4	94.1	92.7	91.5	93.5	93.3	91.5	91.5	91.6	93.7	92.5	92.9	-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Gist-brocades B.V.
(B) STREET: Wateringseweg 1
(C) CITY: Delft
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 2611 XT

(ii) TITLE OF INVENTION: Alkalitolerant Xylanases

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1191 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-47-3
(C) INDIVIDUAL ISOLATE: CBS669.93

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1191

(D) OTHER INFORMATION: /product= "xylanase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG ATT ACA CTT TTT ACA AAG CCT TTT GTT GCT GGA CTA GCG ATC TCT          48
Met Ile Thr Leu Phe Thr Lys Pro Phe Val Ala Gly Leu Ala Ile Ser
 1               5                  10                  15

TTA TTA GTA GGT AGG GGG CTA GGC AAT GTA GCT GCT GCT CAA GGA GGA          96
Leu Leu Val Gly Arg Gly Leu Gly Asn Val Ala Ala Ala Gln Gly Gly
            20                  25                  30

CCA CCA CAA TCT GGA GTC TTT GGA GAG AAT CAC AAA AGA AAT GAT CAG         144
Pro Pro Gln Ser Gly Val Phe Gly Glu Asn His Lys Arg Asn Asp Gln
        35                  40                  45

CCT TTT GCA TGG CAA GTT GCT TCT CTT TCT GAG CGA TAT CAA GAG CAG         192
Pro Phe Ala Trp Gln Val Ala Ser Leu Ser Glu Arg Tyr Gln Glu Gln
    50                  55                  60

TTT GAT ATT GGA GCT CCG GTT GAG CCC TAT CAA TTA GAA GGA AGA CAA         240
Phe Asp Ile Gly Ala Pro Val Glu Pro Tyr Gln Leu Glu Gly Arg Gln
65                  70                  75                  80

GCC CAA ATT TTA AAG CAT CAT TAT AAC AGC CTT GTG GCG GAA AAT GCA         288
Ala Gln Ile Leu Lys His His Tyr Asn Ser Leu Val Ala Glu Asn Ala
                85                  90                  95

ATG AAA CCT GTA TCA CTC CAG CCA AGA GAA GGT GAG TGG AAC TGG GAA         336
Met Lys Pro Val Ser Leu Gln Pro Arg Glu Gly Glu Trp Asn Trp Glu
            100                 105                 110

GGC GCT GAC AAA ATT GTG GAG TTT GCC CGC AAA CAT AAC ATG GAG CTT         384
Gly Ala Asp Lys Ile Val Glu Phe Ala Arg Lys His Asn Met Glu Leu
        115                 120                 125

CGC TTC CAC ACA CTC GTT TGG CAT AGC CAA GTA CCA GAA TGG TTT TTC         432
Arg Phe His Thr Leu Val Trp His Ser Gln Val Pro Glu Trp Phe Phe
    130                 135                 140

ATC GAT GAA AAT GGC AAT CGG ATG GTT GAT GAA ACC GAT CCA GAA AAA         480
Ile Asp Glu Asn Gly Asn Arg Met Val Asp Glu Thr Asp Pro Glu Lys
145                 150                 155                 160
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CGT AAA GCG AAT AAA CAA TTG TTA TTG GAG CGA ATG GAA AAC CAT ATT      528
Arg Lys Ala Asn Lys Gln Leu Leu Leu Glu Arg Met Glu Asn His Ile
                165                 170                 175

AAA ACG GTT GTT GAA CGT TAT AAA GAT GAT GTG ACT TCA TGG GAT GTG      576
Lys Thr Val Val Glu Arg Tyr Lys Asp Asp Val Thr Ser Trp Asp Val
            180                 185                 190

GTG AAT GAA GTT ATT GAT GAT GGC GGG GGC CTC CGT GAA TCA GAA TGG      624
Val Asn Glu Val Ile Asp Asp Gly Gly Gly Leu Arg Glu Ser Glu Trp
        195                 200                 205

TAT CAA ATA ACA GGC ACT GAC TAC ATT AAG GTA GCT TTT GAA ACT GCA      672
Tyr Gln Ile Thr Gly Thr Asp Tyr Ile Lys Val Ala Phe Glu Thr Ala
    210                 215                 220

AGA AAA TAT GGT GGT GAA GAG GCA AAG CTG TAC ATT AAT GAT TAC AAC      720
Arg Lys Tyr Gly Gly Glu Glu Ala Lys Leu Tyr Ile Asn Asp Tyr Asn
225                 230                 235                 240

ACC GAA GTA CCT TCT AAA AGA GAT GAC CTT TAC AAC CTG GTG AAA GAC      768
Thr Glu Val Pro Ser Lys Arg Asp Asp Leu Tyr Asn Leu Val Lys Asp
                245                 250                 255

TTA TTA GAG CAA GGA GTA CCA ATT GAC GGG GTA GGA CAT CAG TCT CAT      816
Leu Leu Glu Gln Gly Val Pro Ile Asp Gly Val Gly His Gln Ser His
            260                 265                 270

ATC CAA ATC GGC TGG CCT TCC ATT GAA GAT ACA AGA GCT TCT TTT GAA      864
Ile Gln Ile Gly Trp Pro Ser Ile Glu Asp Thr Arg Ala Ser Phe Glu
        275                 280                 285

AAG TTT ACG AGT TTA GGA TTA GAC AAC CAA GTA ACT GAA CTA GAC ATG      912
Lys Phe Thr Ser Leu Gly Leu Asp Asn Gln Val Thr Glu Leu Asp Met
    290                 295                 300

AGT CTT TAT GGC TGG CCA CCG ACA GGG GCC TAT ACC TCT TAT GAC GAC      960
Ser Leu Tyr Gly Trp Pro Pro Thr Gly Ala Tyr Thr Ser Tyr Asp Asp
305                 310                 315                 320

ATT CCA GAA GAG CTT TTT CAA GCT CAA GCA GAC CGT TAT GAT CAG TTA     1008
Ile Pro Glu Glu Leu Phe Gln Ala Gln Ala Asp Arg Tyr Asp Gln Leu
                325                 330                 335

TTT GAG TTA TAT GAA GAA TTA AGC GCT ACT ATC AGT AGT GTA ACC TTC     1056
```

```
Phe Glu Leu Tyr Glu Glu Leu Ser Ala Thr Ile Ser Ser Val Thr Phe
                340                 345                 350

TGG GGA ATT GCT GAT AAC CAT ACA TGG CTT GAT GAC CGC GCT AGA GAG      1104
Trp Gly Ile Ala Asp Asn His Thr Trp Leu Asp Asp Arg Ala Arg Glu
        355                 360                 365

TAC AAT AAT GGA GTA GGG GTC GAT GCA CCA TTT GTT TTT GAT CAC AAC      1152
Tyr Asn Asn Gly Val Gly Val Asp Ala Pro Phe Val Phe Asp His Asn
    370                 375                 380

TAT CGA GTG AAG CCT GCT TAC TGG AGA ATT ATT GAT TAA                  1191
Tyr Arg Val Lys Pro Ala Tyr Trp Arg Ile Ile Asp
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Ile Thr Leu Phe Thr Lys Pro Phe Val Ala Gly Leu Ala Ile Ser
 1               5                  10                  15

Leu Leu Val Gly Arg Gly Leu Gly Asn Val Ala Ala Ala Gln Gly Gly
            20                  25                  30

Pro Pro Gln Ser Gly Val Phe Gly Glu Asn His Lys Arg Asn Asp Gln
        35                  40                  45

Pro Phe Ala Trp Gln Val Ala Ser Leu Ser Glu Arg Tyr Gln Glu Gln
    50                  55                  60

Phe Asp Ile Gly Ala Pro Val Glu Pro Tyr Gln Leu Glu Gly Arg Gln
65                  70                  75                  80

Ala Gln Ile Leu Lys His His Tyr Asn Ser Leu Val Ala Glu Asn Ala
```

85 90 95

Met Lys Pro Val Ser Leu Gln Pro Arg Glu Gly Glu Trp Asn Trp Glu
100 105 110

Gly Ala Asp Lys Ile Val Glu Phe Ala Arg Lys His Asn Met Glu Leu
115 120 125

Arg Phe His Thr Leu Val Trp His Ser Gln Val Pro Glu Trp Phe Phe
130 135 140

Ile Asp Glu Asn Gly Asn Arg Met Val Asp Glu Thr Asp Pro Glu Lys
145 150 155 160

Arg Lys Ala Asn Lys Gln Leu Leu Leu Glu Arg Met Glu Asn His Ile
165 170 175

Lys Thr Val Val Glu Arg Tyr Lys Asp Asp Val Thr Ser Trp Asp Val
180 185 190

Val Asn Glu Val Ile Asp Asp Gly Gly Gly Leu Arg Glu Ser Glu Trp
195 200 205

Tyr Gln Ile Thr Gly Thr Asp Tyr Ile Lys Val Ala Phe Glu Thr Ala
210 215 220

Arg Lys Tyr Gly Gly Glu Glu Ala Lys Leu Tyr Ile Asn Asp Tyr Asn
225 230 235 240

Thr Glu Val Pro Ser Lys Arg Asp Asp Leu Tyr Asn Leu Val Lys Asp
245 250 255

Leu Leu Glu Gln Gly Val Pro Ile Asp Gly Val Gly His Gln Ser His
260 265 270

Ile Gln Ile Gly Trp Pro Ser Ile Glu Asp Thr Arg Ala Ser Phe Glu
275 280 285

Lys Phe Thr Ser Leu Gly Leu Asp Asn Gln Val Thr Glu Leu Asp Met
290 295 300

Ser Leu Tyr Gly Trp Pro Pro Thr Gly Ala Tyr Thr Ser Tyr Asp Asp
305 310 315 320

Ile Pro Glu Glu Leu Phe Gln Ala Gln Ala Asp Arg Tyr Asp Gln Leu

325 330 335

Phe Glu Leu Tyr Glu Glu Leu Ser Ala Thr Ile Ser Ser Val Thr Phe
340 345 350

Trp Gly Ile Ala Asp Asn His Thr Trp Leu Asp Asp Arg Ala Arg Glu
355 360 365

Tyr Asn Asn Gly Val Gly Val Asp Ala Pro Phe Val Phe Asp His Asn
370 375 380

Tyr Arg Val Lys Pro Ala Tyr Trp Arg Ile Ile Asp
385 390 395

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: FA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CACACKCTKG TKTGGCA 17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: FB

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CATACKTTKG TTTGGCA 17

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: FR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TMGTTKACMA CRTCCCA 17

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: GAF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GARTAYTAYA THGTNGA 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: GAR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CKNACNGACC ARTA 14

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: GBF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GARTAYTAYG TNGTNGA 17

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: GBR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CKNACRCTCC ARTA 14

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: GCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCRCTRCTKT GRTANCCYTC 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 142 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-43-3
(C) INDIVIDUAL ISOLATE: CBS672.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CATAGCCAAG TACCTGAATG GTTTTTCATC GATAAAGACG GTAATCGTAT GGTAGATGAA 60

ACAAATCCAG CGAAACGTGA GGCTAATAAA CAGCTTTTAT TAGAGCGGAT GGAAACACAT 120

ATCAAAACGG TTGTGGAACG TT 142

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-47-3
(C) INDIVIDUAL ISOLATE: CBS669.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CACACGCTGG TTTGGCATAG CCAAGTACCA GAATGGTTTT TCATCGATGA AAATGGCAAT 60

CGGATGGTTG ATGAAACCGA TCCAGAAAAA CGTAAAGCGA ATAAACAATT GTTATTGGAG 120

CGAATGGAAA ACCATATTAA AACGGTTGTT GAACGTTATA AAGATGATGT GACTTCATGG 180

GACGTGGTAA ACGA 194

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 2-26-2
(C) INDIVIDUAL ISOLATE: CBS673.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CACACGCTGG TTTGGCACAG CCAAGTACCA GAATGGTTTT TCATCGATGA AGACGGCAAT 60

CGGATGGTGG ATGAAACAGA CCCAGATAAA CGTGAAGCGA ATAAACAGCT GTTATTGGAG 120

CGCATGGAAA ACCATATTAA AACGGTTGTT GAACGTTATA AAGATGATGT GACTTCATGG 180

GACGTGGTCA ACGA 194

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 2-m-1
(C) INDIVIDUAL ISOLATE: CBS667.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CACACTCTTG TTTGGCATAG CCAAGTACCA GAATGGTTTT TCATCGATGA AAATGGCAAT 60

CGGATGGTTG ATGAAACCGA TCCAGAAAAA CGTAAAGCGA ATAAACAATT GTTATTGGAG 120

CGAATGGAAA ACCATATTAA AACGGTTGTT GAACGTTATA AAGATGATGT GACTTCATGG 180

GACGTGGTAA ACGA 194

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-25-2
(C) INDIVIDUAL ISOLATE: CBS671.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GAATATTATA TTGTCGACAG TTGGGGCAAC TGGCGTCCAC CAGGAGCAAC GCCTAAGGGA 60

ACCATCACTG TTGATGGAGG AACATATGAT ATCTATGAAA CTCTTAGAGT CAATCAGCCC 120

TCCATTAAGG GGATTGCCAC ATTTAAACAA TATTGGAGCG TCCG 164

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-43-3
(C) INDIVIDUAL ISOLATE: CBS672.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAATATTATA TTGTCGACAG TTGGGGCAAC TGGCGTCCAC CAGGAGCAAC GCCTAAGGGA 60

ACCATCACTG TTGATGGAGG AACATATGAT ATCTATGAAA CTCTTAGAGT CAATCAGCCC 120

TCCATTAAGG GGATTGCCAC ATTTAAACAA TATTGGAGCG TCCG 164

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-43-3
(C) INDIVIDUAL ISOLATE: CBS672.93

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GAATATTACA TCGTTGATAG CTGGGGAAGC TGGCGTCCAC CAGGAGCTAA CGCAAAAGGA 60

ACGATTACTG TTGACGGTGG TGTTTACGAT ATTTATGAAA CAACTCGAGT TAACCAACCT 120

TCCATTATTG GAGATGCGAC TTTCCAACAG TACTGGAGTG TGCG 164

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 744 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: 1-43-3
(C) INDIVIDUAL ISOLATE: CBS672.93

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..744
(D) OTHER INFORMATION: /product= "xylanase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
ATG AGC CAA AAG AAA TTG ACG TTG ATT AAC CTT TTT AGT TTG TTT GCA      48
Met Ser Gln Lys Lys Leu Thr Leu Ile Asn Leu Phe Ser Leu Phe Ala
 1               5                  10                  15

CTA ACC TTA CCT GCA AGA ATA AGT CAG GCA CAA ATC GTC ACC GAC AAT      96
Leu Thr Leu Pro Ala Arg Ile Ser Gln Ala Gln Ile Val Thr Asp Asn
            20                  25                  30

TCC ATT GCC ACC CGC GGT GGT TAT GAT TAT GAA TTT TGG AAA GAT AGC     144
Ser Ile Ala Thr Arg Gly Gly Tyr Asp Tyr Glu Phe Trp Lys Asp Ser
        35                  40                  45

GGT GGC TCT GGG ACA ATG ATT CTC AAT CAT GGC GGT ACG TTC AGT GCC     192
Gly Gly Ser Gly Thr Met Ile Leu Asn His Gly Gly Thr Phe Ser Ala
    50                  55                  60

CAA TGG AAT AAT GTT AAC AAT ATA TTA TTC CGT AAA GGT AAA AAA TTC     240
Gln Trp Asn Asn Val Asn Asn Ile Leu Phe Arg Lys Gly Lys Lys Phe
65                  70                  75                  80

AAT GAA ACA CAA ACA CAC CAA CAA GTT GGT AAC ATG TCC ATA AAC TAT     288
Asn Glu Thr Gln Thr His Gln Gln Val Gly Asn Met Ser Ile Asn Tyr
                85                  90                  95

GGC GCA AAC TTC CAG CCA AAC GGT AAT GCG TAT TTA TGC GTC TAT GGT     336
Gly Ala Asn Phe Gln Pro Asn Gly Asn Ala Tyr Leu Cys Val Tyr Gly
            100                 105                 110

TGG ACT GTT GAC CCT CTT GTT GAA TAT TAT ATT GTC GAC AGT TGG GGC     384
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Trp Thr Val Asp Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly
        115                 120                 125

AAC TGG CGT CCA CCA GGA GCA ACG CCT AAG GGA ACC ATC ACT GTT GAT      432
Asn Trp Arg Pro Pro Gly Ala Thr Pro Lys Gly Thr Ile Thr Val Asp
    130                 135                 140

GGA GGA ACA TAT GAT ATC TAT GAA ACT CTT AGA GTC AAT CAG CCC TCC      480
Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Leu Arg Val Asn Gln Pro Ser
145                 150                 155                 160

ATT AAG GGG ATT GCC ACA TTT AAA CAA TAT TGG AGT GTC CGA AGA TCG      528
Ile Lys Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Ser
                165                 170                 175

AAA CGC ACG AGT GGC ACA ATT TCT GTC AGC AAC CAC TTT AGA GCG TGG      576
Lys Arg Thr Ser Gly Thr Ile Ser Val Ser Asn His Phe Arg Ala Trp
            180                 185                 190

GAA AAC TTA GGG ATG AAC ATG GGG AAA ATG TAT GAA GTC GCG CTT ACT      624
Glu Asn Leu Gly Met Asn Met Gly Lys Met Tyr Glu Val Ala Leu Thr
        195                 200                 205

GTA GAA GGC TAT CAA AGT AGC GGA AGT GCT AAT GTA TAT AGC AAT ACA      672
Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val Tyr Ser Asn Thr
    210                 215                 220

CTA AGA ATT AAC GGA AAC CCT CTC TCA ACT ATT AGT AAT AAC GAG AGC      720
Leu Arg Ile Asn Gly Asn Pro Leu Ser Thr Ile Ser Asn Asn Glu Ser
225                 230                 235                 240

ATA ACT CTA GAT AAA AAC AAT TAG                                     744
Ile Thr Leu Asp Lys Asn Asn
                245
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 247 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Met Ser Gln Lys Lys Leu Thr Leu Ile Asn Leu Phe Ser Leu Phe Ala
1 5 10 15

Leu Thr Leu Pro Ala Arg Ile Ser Gln Ala Gln Ile Val Thr Asp Asn
20 25 30

Ser Ile Ala Thr Arg Gly Gly Tyr Asp Tyr Glu Phe Trp Lys Asp Ser
35 40 45

Gly Gly Ser Gly Thr Met Ile Leu Asn His Gly Gly Thr Phe Ser Ala
50 55 60

Gln Trp Asn Asn Val Asn Asn Ile Leu Phe Arg Lys Gly Lys Lys Phe
65 70 75 80

Asn Glu Thr Gln Thr His Gln Gln Val Gly Asn Met Ser Ile Asn Tyr
85 90 95

Gly Ala Asn Phe Gln Pro Asn Gly Asn Ala Tyr Leu Cys Val Tyr Gly
100 105 110

Trp Thr Val Asp Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly
115 120 125

Asn Trp Arg Pro Pro Gly Ala Thr Pro Lys Gly Thr Ile Thr Val Asp
130 135 140

Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Leu Arg Val Asn Gln Pro Ser
145 150 155 160

Ile Lys Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Ser
165 170 175

Lys Arg Thr Ser Gly Thr Ile Ser Val Ser Asn His Phe Arg Ala Trp
180 185 190

Glu Asn Leu Gly Met Asn Met Gly Lys Met Tyr Glu Val Ala Leu Thr
195 200 205

Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val Tyr Ser Asn Thr
210 215 220

Leu Arg Ile Asn Gly Asn Pro Leu Ser Thr Ile Ser Asn Asn Glu Ser
225 230 235 240

Ile Thr Leu Asp Lys Asn Asn
245

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1521 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus sp.
(C) INDIVIDUAL ISOLATE: DSM 8721

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC GCAGGAAGCC GGCGGATCCC 60

TTCGGGGTGA ANCCGGTGGA ATGAGCGGCG GACGGGTGAG TAACACGTGG GCAACCTACC 120

TTGTAGACTG GGATAACTCC GGGAAACCGG GGCTAATACC GGATGATCAT TTGGATCGCA 180

TGATCCGAAT GTAAAAGTGG GGATTTATCC TCACACTGCA AGATGGGCCC GCGGCGCATT 240

AGCTAGTTGG TAAGGTAATG GCTTACCAAG GCGACGATGC GTAGCCGACC TGAGAGGGTG 300

ATCGGCCACA CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC AGTAGGGAAT 360

CATCCGCAAT GGGCGAAAGC CTGACGGTGC AACGCCGCGT GAACGATGAA GGTTTTCGGA 420

TCGTAAAGTT CTGTTATGAG GGAAGAACAA GTGCCGTTCG AATAGGTCGG CACCTTGACG 480

GTACCTCACG AGAAAGCCCC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT ACGTAGGGGG 540

CAAGCGTTGT CCGGAATTAT TGGGCGTAAA GCGCGCGCAG GCGGTCTCTT AAGTCTGATG 600

TGAAAGCCCA CGGCTCAACC GTGGAGGGTC ATTGGAAACT GGGGGACTTG AGTGTAGGAG 660

AGGAAAGTGG AATTCCACGT GTAGCGGTGA AATGCGTAGA TATGTGGAGG AACACCAGTG 720

GCGAAGGCGA CTTTCTGGCC TACAACTGAC GCTGAGGCGC GAAAGCGTGG GGAGCAAACA 780

GGATTAGATA CCCTGGTAGT CCACGCCGTA AACGATGAGT GCTAGGTGTT AGGGGTTTCG 840

ATACCCTTAG TGCCGAAGTT AACACATTAA GCACTCCGCC TGGGGAGTAC GGCCGCAAGG 900

CTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCAGT GGAGCATGTG GTTTAATTCG 960

AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTCTG ACACCTCTGG AGACAGAGCG 1020

TTCCCCTTCG GGGGACAGAG TGACAGGTGG TGCATGGTTG TCGTCAGCTC GTGTCGTGAG 1080

ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTGATCT TAGTTGCCAG CATTCAGTTG 1140

GGCACTCTAA GGTGACTGCC GGTGATAAAC CGGAGGAAGG TGGGGATGAC GTCAAATCAT 1200

CATGCCCCTT ATGACCTGGG CTACACACGT GCTACAATGG ATGGTACAAA GGGCAGCGAG 1260

ACCGCGAGGT TAAGCGAATC CCATAAAGCC ATTCTCAGTT CGGATTGCAG GCTGCAACTC 1320

GCCTGCATGA AGCCGGAATT GCTAGTAATC GCGGATCAGC ATGCCGCGGT GAATACGTTC 1380

CCGGGTCTTG TACACACCGC CCGTCACACC ACGAGAGTTT GTAACACCCG AAGTCGGTGC 1440

GGTAACCTTT TGGAGCCAGC CGNCGAAGGT GGGACAGATG ATTGGGGTGA AGTCGTAACA 1500

AGGTATCCCT ACCGGAAGGT G 1521

Claims

1. A xylanase having considerable activity at pH 9.0 and at a temperature of 70°C.

2. A xylanase according to claim 1, and characterized in that the xylanase is obtainable from a microorganism of which the 16S ribosomal DNA sequence shares more than 92 % identity with the 16S ribosomal DNA sequence of strain DSM 8721 as listed in SEQ ID NO 20.

3. A xylanase according to claims 1 or 2, and characterized in that the xylanase is obtainable from the Bacillus species DSM 8721.

4. A xylanase according to any one of claims 1 to 3 and characterized in that the amino acid sequence of the xylanase shares more than 72 % identity with the amino acid sequence as listed in SEQ ID NO 19.

5. A xylanase having considerable activity at pH 9.0 and at a temperature of 70°C, and characterized in that the xylanase is obtainable from a microorganism selected from the group consisting of the strains deposited under the following deposition numbers: CBS 666.93, 667.93, 669.93, and 673.93.

6. A xylanase according to claim 5 and further characterized in that the amino acid sequence of the xylanase shares more than 93 % identity with the amino acid sequence as listed in SEQ ID NO 2.

7. A xylanase having considerable activity at pH 9.0 and a temperature of 70°C, and characterized in that the xylanase produces an increase in % ISO brightness of soft-wood pulp over non-enzymatically treated pulp of at least 1.0, preferably an increase in % ISO brightness of soft-wood pulp

between 1.5 and 5.0, in an ECF pulp bleaching process wherein the enzyme treatment of the pulp is carried out at a pH of 9.0 at a temperature of 65°C.

8. A xylanase having considerable activity at pH 9.0 and a temperature of 70°C, and characterized in that the xylanase produces an increase in % ISO brightness of soft-wood pulp over non-enzymatically treated pulp of at least 1.0, preferably an increase in % ISO brightness of hard-wood pulp between 1.2 and 3.0, in an ECF pulp bleaching process wherein the enzyme treatment of the pulp is carried out at a pH of 9.0 at a temperature of 65°C.

9. An isolated DNA sequence encoding a xylanase according to any one of claims 1 to 8.

10. A vector capable of transforming a microbial host cell and characterized in that the vector comprises a DNA sequence according to claim 9.

11. A vector according to claim 10 and characterized in that the DNA sequence is operably linked to expression signals that ensure the expression of the DNA sequence in the microbial host.

12. A microbial host which contains a vector according to claims 10 or 11.

13. A microbial host according to claim 12 and characterized in that the microbial host expresses the DNA sequence.

14. A process for the preparation of the xylanases according to any of claims 1 to 8 and characterized in that the xylanase is obtainable by cultivation of a microorganism producing the xylanases in a suitable medium, followed by recovery of the xylanases.

15. A process according to claim 14 wherein the microorganisms is a microbial host according to claim 13.

16. A process for degradation of xylan comprising the use of the xylanases according to any one of claims 1 to 8.

17. A process for delignifying wood pulp comprising the use of the xylanases according to any one of claims 1 to 8.

18. A process for the bleaching of pulp comprising the use of the xylanases according to any one of claims 1 to 6.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 94/04312

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/24 C12N15/55 D21H17/00 //(C12N15/55,C12R1:07)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol.51, no.3, 1987, TOKYO JP pages 953 - 955 T.HAMAMOTO ET AL. 'Nucleotide...'	1-6,9-16
Y	see the whole document	7,8,17, 18

X	METHODS IN ENZYMOLOGY, vol.160, 1988, NEW YORK US pages 655 - 659 T.AKIBA ET AL. 'XYLANASE...' see the whole document	1,16

X	BIOTECHNOLOGY LETTERS, vol.14, no.11, 1992 pages 1045 - 1046 N.GUPTA ET AL. 'A THERMOSTABLE...' see the whole document	1,16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 May 1995	11 -05- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ENZYME AND MICROBIAL TECHNOLOGY, vol.8, 1986, HAYWARDS HEATH GB pages 309 - 314 H.GRUNINGER ET AL. 'A NOVEL , HIGHLY THERMOSTABEL...' see the whole document	1,16
Y	APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol.40, no.1, October 1993, BERLIN DE pages 57 - 62 LEA BEZAZEL ET AL. 'CHARACTERIZATION AND DELIGNIFICATION...' see the whole document	17
Y	CHEMICAL ABSTRACTS, vol. 120, no. 4, 24 January 1994, Columbus, Ohio, US; abstract no. 33174j, SHOHAM, YUVAL ET AL 'Delignification of wood pulp by a thermostable xylanase from Bacillus stearothermophilus strain T-6' see abstract & BIODEGRADATION, 3(2-3), 207-18, 1992	7,8,17, 18
Y	WO,A,91 18976 (NOVO NORDISK A/S) 12 December 1991 see claims 1-13,25-34	7,8,17, 18
X,P	BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, vol.58, no.1, 1994, JP pages 78 - 81 S.NAKAMURA ET AL. 'Thermophillic...' see the whole document	1,5,7-18

1

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9118976	12-12-91	NONE	